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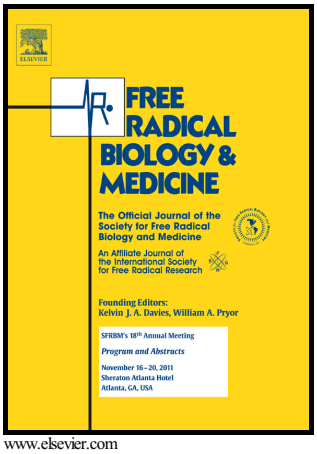

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A discrete role for alternative oxidase under hypoxia to increase nitric oxide and drive energy production☆

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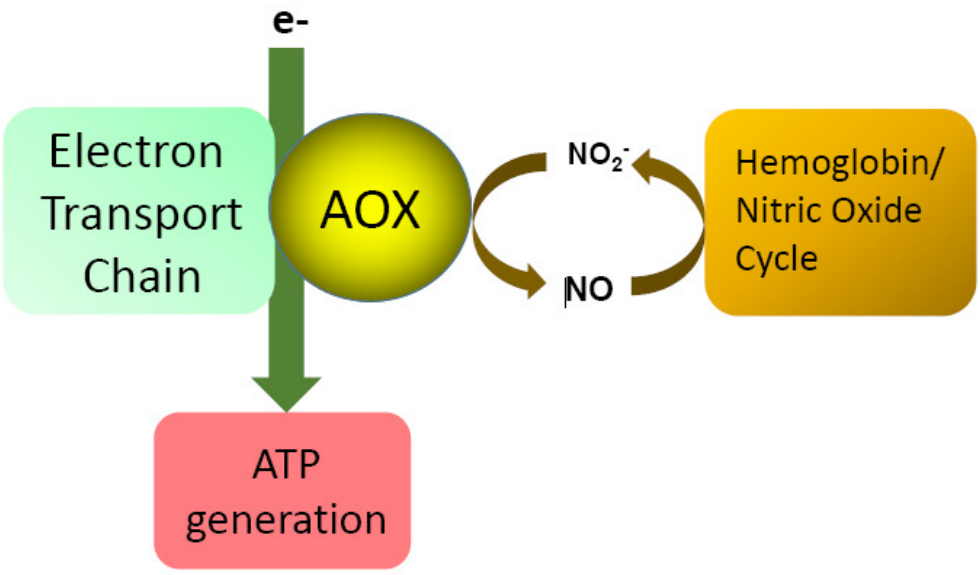
☆ **Once sentence summary**

Under oxygen limiting conditions AOX can reduce nitrite to NO thereby it can drive Hb-NO cycle to increase energy efficiency

Abstract

Alternative oxidase (AOX) is an integral part of the mitochondrial electron transport and can prevent reactive oxygen species (ROS) and nitric oxide (NO) production under non-stressed, normoxic conditions. Here we assessed the roles of AOX by imposing stress under normoxia in comparison to hypoxic conditions using AOX over expressing (AOX OE) and anti-sense (AOX AS) transgenic Arabidopsis seedlings and roots. Under normoxic conditions stress was induced with the defence elicitor flagellin (flg22). AOX OE reduced NO production whilst this was increased in AOX AS. Moreover AOX AS also exhibited an increase in superoxide and therefore peroxynitrite, tyrosine nitration suggesting that scavenging of NO by AOX can prevent toxic peroxynitrite formation under normoxia. In contrast, during hypoxia interestingly we found that AOX is a generator of NO. Thus, the NO produced during hypoxia, was enhanced in AOX OE and suppressed in AOX AS. Additionally, treatment of WT or AOX OE with the AOX inhibitor SHAM inhibited hypoxic NO production. The enhanced levels of NO correlated with expression of non-symbiotic haemoglobin, increased NR activity and ATP production. The ATP generation was suppressed in *nia1,2* mutant and non symbiotic haemoglobin antisense line treated with SHAM. Taken together these results suggest that hypoxic NO generation mediated by AOX has a discrete role by feeding into the haemoglobin-NO cycle to drive energy efficiency under conditions of low oxygen tension.

Graphical abstract



Abbreviations

Alternative oxidase 1a (AOX1a); Aminophenyl fluorescein (APF); cycle threshold (CT); 4-amino-5-methylamino 2',7'-difluorofluorescein diacetate (DAF-FM-DA); 3,3-diaminobenzidine (DAB); flagellin (flg22); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); nitric oxide (NO); nitroblue tetrazolium (NBT); peroxynitrite ($ONOO^-$); phenylmethylsulfonyl fluoride (PMSF); salicylhydroxamic acid (SHAM); tris-buffered saline (TBS).

Keywords: alternative oxidase, nitric oxide, peroxynitrite, superoxide, energy efficiency

1. Introduction

Mitochondria are responsible for generating most of the ATP by using proton motive force generated via electron transport chain (ETC) during the transfer of electrons from reducing equivalents to oxygen. As well as the usual terminal oxidase, cytochrome c oxidase (COX), plant mitochondria also contain an alternative oxidase (AOX). The AOX accepts electrons from ubiquinone pool and thus bypasses the later proton pumping steps in the electron transport chain, leading to reduced generation of ATP. Electron transfer via AOX can reduce production of ROS, contributing to the prevention of oxidative stress [1].

Nitric oxide (NO) production is a recently emerged feature of mitochondria via mechanisms that is still under investigation [2]. More widely, NO is synthesised by several reductive and oxidative pathways in plants. Cytosolic nitrate reductase (cNR), plasma membrane-bound nitrite reductase (PM NI-NOR) and mitochondrial electron transport produce NO usually from the reduction of NO_2^-. However, nitric oxide synthase-like activity, polyamine and hydroxylamine mediated NO pathways are oxidative in nature [3]. Plant mitochondria produce very high levels of NO (in the range 1-20 nmoles/gFW/hr) only under hypoxic conditions using nitrite as a terminal electron acceptor [4]. This, subcellular location of NO synthesis in mitochondria, is important for its involvement in cell death, hypoxia tolerance and also processes such as retrograde signalling [2]. The NO production is sensitive to myxothiazol (Myxo) and cyanide which inhibit complex III and COX respectively. The K_m (nitrite) for this nitrite:NO reductase reaction is 175 µM and *K*i 0.05% O_2 for NO production [4] suggesting NO production takes place under very low oxygen conditions. Alber et al.[5] defined a novel mitochondrial NO which proved that NO_2^- was being reduced at the ubiquinol oxidation centre (P, Qo site).

It is also the case that mitochondria are scavengers of NO [6] particularly under normoxic conditions [7]. Transgenic manipulation of AOX revealed a reciprocal relationship between AOX protein expression and reactive oxygen species (ROS) generation in cell cultures [8] and NO in leaves [7] of tobacco. Cvetkovska & Vanlerberghe, [7] found that antisense tobacco with reduced levels of AOX had elevated levels of O_2^- and NO suggesting that the AOX respiratory pathway decreases leakage from the ETC. AOX reduces electron flow through complexes III and IV (COX) and thus decreases the leakage of electrons to nitrite and suppressed the accumulation of NO.

Scavenging of NO is very important as excess of NO can react rapidly with proteins and other free radicals, leading to the formation of S-nitrosylated proteins and other compounds, tyrosine-nitrated proteins and the production of nitrite and peroxynitrite ($ONOO^-$). Mitochondrially produced NO can also be scavenged by other systems, e.g. NO diffusion into the cytosol leads to the conversion of NO to nitrate by cytosolic non-symbiotic haemoglobin [9]. Nitrate is then converted to nitrite by nitrate reductase, and the whole sequence of reactions is known as the haemoglobin/nitric oxide (Hb/NO) cycle. This cycle can lead to limited ATP generation [10].

The interactions between AOX and NO production have also been investigated. Cvetkovska & Vanlerberghe [7] demonstrated that transgenic tobacco plants that lack AOX exhibited increased levels of O_2^- and NO. This led the authors to conclude that the AOX respiratory pathway prevented ROS and NO production by dampening electron flow from the ETC to oxygen or nitrite at COX. This suggests that AOX can prevent excess NO production under normoxia, but the role of AOX under stress conditions is not yet known. Alber et al. [5] have shown that AOX contributed to net rates of NO generated by the electron transport chain. It is therefore interesting that

NO is an inducer for AOX [11]. This suggests a mutually regulatory mechanism whereby inducing AOX, NO can control both ROS and NO homeostasis. This mechanism is likely to be especially useful when NO and ROS levels increase under stress conditions. For instance, Huang et al. [12] found that AOX induction takes place in the presence of the bacterial elicitor harpin due to NO. In another study (Fu et al.) [13] showed AOX induction by NO protected plants from tobacco mosaic virus (TMV). There is clear evidence that mitochondrially produced NO can also nitrosylate proteins such as glycine decarboxylase (GDC) and thus promote cell death by countering AOX mediated protection [14].

In further considering the interactions between AOX and NO, it is clear that this needs to be further characterised with stress under normoxic conditions and equally, under hypoxic conditions. This latter situation is of great interest due to its occurrence at low oxygen tensions which is known to produce very high levels of NO and is seldom considered in investigations focusing on AOX. In the present study, we confirmed the AOX role in scavenging of NO and ROS in stress elicited under normoxia. However, under hypoxia we demonstrate a novel role for AOX in the production of NO that is linked to haemoglobin-NO cycle to increase energy efficiency. We further found that higher levels of NO production mediated by AOX under hypoxia does not contribute to toxic peroxynitrite formation.

2. Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (AOX OE and AOX AS and NR null mutant nia 1,2) lines were obtained from the Nottingham Arabidopsis Stock Centre (UK), N6591,N6707,N 2356

respectively. The Silencing line of nHb1 was described in [31]. Seeds were surface sterilized using 75% ethanol and 0.1% triton X-100 followed by washing with 100% ethanol. Seeds were allowed to air dry and grown on half-MS agar plates (supplemented with 1.5% sucrose). Initially, plates were kept at 4 °C for two days for stratification and later transferred to a growth chamber. The growth chamber had an 8h light/ 16 h dark photoperiod with temperatures of 24/20 °C, in each respective light regime. The light intensity was 50-100 µmol $m^{-2}s^{-1}$.

Treatments

Two weeks old plants were used for treatments. For normoxia, plants were sampled directly from growth chamber. For hypoxia treatment, plants were subjected to 0.4% oxygen in a closed container (BiOxia H-800, Mumbai India) for 6 h.. Additionally, to induce the NO production under normoxic condition, 2.5 µM flg22 were directly added to seedlings and incubated for 10 min at room temperature. To inhibit the AOX pathway, seedlings were treated with 2.5 mM salicylhydroxamic acid (SHAM). After treatment, samples were either immediately used for fluorescence studies, or alternatively tissues were stored at -80 °C for other experiments.

Measurements of NO, $ONOO^-$, O_2^- and $H_2O_2^-$

For NO and $ONOO^-$ detection, roots were incubated in 10 mM HEPES pH 7.2 containing 10 µM DAF-FM or 10 µM APF dye respectively, for 20 min at room temperature under dark conditions. The unbound dye was washed away with 10 mM HEPES pH 7.2 buffer. The images were taken in fluorescence microscope using λ_{488} excitation and $\lambda_{500\text{-}530}$ emission wavelength and 2 s light exposure and 20X zoom. . For APF quantification 3-4 seedlings weighing total 25 mg were incubated with 10

μM APF for 20 min in dark following treatments. Seedlings were crushed with micro pestle in 500 μl of extraction buffer that containing 50 mM HEPES, pH 7.2 and centrifuged at 13000g for 10 min and fluorescence from supernatant was quantified by microplate reader (Polar Star Omega, BMG Labtech, Germany) at λ_{488} excitation and $\lambda_{500\text{-}530}$ emission. The plate was shaken before each measurement. Peroxynitrite-related fluorescence was calculated by subtracting the auto fluorescence value of seedlings (not incubated with APF dye) from total fluorescence of APF treated seedlings. Three biological and 3 technical replicate were used for each treatment.

For O_2^- detection, roots were incubated with 0.1% NBT solution [40] for 24 h at room temperature under dark conditions. For H_2O_2 detection, roots were incubated with 0.1% DAB solution [40] for 2 h at room temperature under dark condition. The unbound dye was washed away with 70% ethanol and stored in the same until imaged. Images were taken under bright field using 20X zoom and 83 ms light exposure. The extent of staining was quantified using Image J software for 20 plants per treatment.

RNA isolation

Total RNA was isolated from seedlings using Trizol reagent according to manufacturer's instructions (Invitrogen). Briefly, 100 mg seedlings were ground in liquid nitrogen as fine powder and 1 ml of TRI reagent was added. Subsequently, the mixture was vortexed and incubated for 10 min at 25 °C. Thereafter, 200 μl of chloroform were added and incubated for 5 min at 25 °C. After vortexing, samples were centrifuged at 12,000×g for 15 min at 4 °C. The upper aqueous phase containing total RNA was transferred into a fresh tube containing an equal volume of

isopropanol. Mixtures were incubated for 1h at -80 °C followed by centrifugation as described above. Supernatants were discarded and the precipitated RNA pellets were washed in 1 ml of 75% ethanol at 7500×g for 5 min at 4 °C. The resulting pellets were allowed to air-dry for 10-15 min and then resuspended in sterile DEPC-treated water The obtained RNA was treated with RNase-free DNase I (1U/µg RNA) (Promega) and incubated for 30 min at 30 °C. Thereafter, 1 µl of RQ1DNase solution was added to terminate the reaction followed by incubating for 10 min at 65 °C to inactivate the DNase.

Real time PCR

Total RNA equivalent to 2 µg was taken for cDNA synthesis and synthesized according to the kit manufacturer's (Applied Biosystem) instructions. Real-time PCR was carried out at 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s using 7900 Fast Real-Time PCR machine (Applied Biosystems). The *18S rRNA* was used as a housekeeping gene for normalisation purposes. Cycle threshold (CT) values were obtained from the exponential phase of PCR amplification. The comparative CT method was used to analyze the results [41]. In this method, genes of interest (*GOI*) were normalized against *18S rRNA* (housekeeping gene) expression, generating a ΔCT value (ΔCT = *GOI* CT−*18S rRNA* CT). The relative expression was then calculated according to the equation $2^{-\Delta\Delta CT}$ and normoxia was used as a calibrator. Primer details are given in SupplimentaryTable 1.

SDS-PAGE and western blotting

Total protein was extracted from frozen tissues. Extracts were prepared by grinding tissue in extraction buffer (50 mM potassium phosphate buffer pH 7.5 and 1 mM

phenylmethylsulfonyl fluoride) at 4°C. Homogenate was centrifuged at 14,000×g for 15 min at 4°C. The supernatant was recovered, and total protein was estimated using the Bradford protein assay.

Samples were mixed with 4X SDS loading dye and boiled for 3-5 min. Total protein (10, 25 and 50 µg) was separated on 10% SDS-PAGE [42]. Thereafter, protein was transferred to nitrocellulose membranes using iBlot® 2 dry blotting system (Life Technologies) at 20V for 7 min. To block the protein, membrane was incubated in a blocking buffer (1X TBS with 1% nonfat dry milk) for 2 h at 25 °C and then washed three times (5 min each) in TBST (0.1% Tween 20 in 1X TBS). The membrane was incubated overnight at 4 °C with primary antibody i.e. anti-AOX1a (1:1000 dilution, Agrisera) or against 3-nitrotyrosine (1:2000 dilution, Molecular Probes). Next, the membrane was washed three times (10 min each) in TBST followed by incubated for 2 h with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution, Bangalore GeNei™). After that membrane was washed three times in TBST for 10 min each. The blot was developed using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific) and visualized in ChemiDoc (Bio-Rad). Band intensity was quantified by using Image J program.

ATP measurement

Approximately 50 mg tissue was grinded in 0.5 ml of 10% w/v TCA and centrifuged at 10,000×g for 5 min at 4°C. The supernatant was collected and neutralized with 10N KOH. The sample was centrifuged at 10,000×g for 5 min at 4°C. Supernatant was recovered and diluted 1:10 with the buffer provided in ATP determination kit, (Molecular Probes). Diluted sample (equivalent to 0.25 µg protein) was added to 100 µl of reaction mixture and luminescence was recorded for 3 min (ATP

determination kit, Molecular Probe). ATP standard (1-5 nM) was run every time along with unknown samples.

Nitrate reductase activity

NR activity was measured according to [15] with minor modifications. The frozen tissue was ground in liquid nitrogen and extracted in 0.5 ml of extraction buffer (100 mM HEPES pH 7.6, 3.5 mM β-mercaptoethanol, 15 mM $MgCl_2$, 0.5% PVP, 0.5% BSA and 0.3% Triton X-100). The homogenate was centrifuged at 14,000×g for 10 min at 4 °C. The supernatant was recovered and used directly in the assay. The supernatant equivalent to 100 µg protein was added to assay buffer (100 mM HEPES pH 7.6, 1 mM DTT, 15 mM $MgCl_2$, 5 mM KNO_3 and 0.2 mM NADH) in a final volume of 1ml. After 10 min, the reaction was stopped by adding 125 µl of zinc acetate (0.5M). Afterwards, 1 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene dihydrochloride) was added and incubated for 10 min at 25 °C. The absorbance was read at 546 nm. 0.1 µM and 10 µM KNO_2 was used as standard.

Hypoxia tolerance assay

To check the hypoxia tolerance, 20 seeds from each line were germinated on vertical MS plates. After 9 day old seedlings were subjected to 0.4% O_2 for 24 h under dark conditions. The plates were then transferred to light with normal growth conditions to examine their recovery from hypoxia. After a week the phenotypes of surviving and dead plants were assessed.

3. Results

Induction of AOX under normoxia and hypoxia

Cvetkovska & Vanlerberghe [7] indicated that lack of AOX increased levels of superoxide and NO. However, it is not known whether AOX can prevent NO under stress conditions which are not associated with cell death. Therefore we compared normoxic stress imposed by the application of the pathogen-associated molecular pattern (PAMP) flagellin (flg22) to be compared with stress-linked to hypoxia.

Initially, we sought to assess the effect of each form of stress on AOX protein accumulation. We treated WT Col-0 seedlings with flg22 for 10 min under normoxic conditions. flg22 led to a 16 fold induction of *AOX1A* transcript levels and 9 fold increase in protein levels. We then tested the impact of hypoxia on induction of AOX. For this purpose seedlings were flushed continuously for 6 hours with 0.4% oxygen. A seven-fold induction in *AOX1A* transcript levels and 11 fold increase protein levels were observed in response to hypoxia treatment (Fig 1). Taken together these results suggesting that AOX induction occurs in response to flg22 treatment and hypoxia.

AOX is a scavenger of NO under normoxia in response to flg 22 treatment

To measure the impact on AOX on NO elicited with flg22 under normoxia we employed the fluorescent reporter, 4-amino-5-methylamino 2' ,7' - difluorofluorescein diacetate (DAF-FM-DA) (Fig. 2). Under normoxic conditions WT Col-0 roots generated a small amount of NO whereas slightly reduced levels (but not significantly compared to WT) of NO was observed in AOX OE roots. However, a significant increase in NO levels were observed in AS roots, suggesting that AOX can scavenge NO under normoxic conditions. In response to flg22 we observed increased NO generation. This was significantly increased in AOX AS lines but

tended towards a decrease in AOX OE. This suggested a negative correlation between AOX and NO in flg22 induction.

AOX can reduce superoxide, hydrogen peroxide and peroxynitrite in response to flg22 treatment

AOX can prevent over reduction of ubiqunol pool thereby it can prevent excess formation of O_2^-. Excess of NO can react with O_2^- to form $ONOO^-$. As AOX prevents excess of NO (Fig. 2) it can modulate the formation of $ONOO^-$. Hence, parallel to our investigations of flg22-elciited NO effects, we assess its impact on O_2^- and $ONOO^-$. For this purpose Nitro blue tetrazolium (NBT) staining was used to visualize O_2^- levels (Fig. 3). Unchallenged roots of WT and AOX AS line produced slightly higher amount of superoxide under normoxic conditions than AOX OE roots (Fig. 3A). In response to flg22, an increase in O_2^- was found in WT and AOX AS than AOX OE roots. This indicated that AOX can contribute for scavenging of flg22-elicited O_2^- production.

Given this role for NO, we sought to confirm that AOX played a role in controlling $ONOO^-$, therefore, its levels were measured by Aminophenyl fluorescein (APF). Untreated roots of WT, AOX OE and AS produced very low levels of $ONOO^-$, but upon application of flg22, APF fluorescence increased approximately 3 fold in WT and 5 fold in AS line (Fig 3B). AOX OE produced very low levels of $ONOO^-$ suggesting that AOX can modulate $ONOO^-$, most likely though suppression of both NO (Fig. 2) and O_2^- (Fig. 3A). $ONOO^-$ is a strong nitrating agent, hence we checked tyrosine nitration in responses to flg22 (Fig. 3C). Under control conditions the all the genotypes displayed some extent of tyrosine nitration. Upon flg22 treatment Tyr-nitration has increased approximately 2.5 fold in WT, whereas a significant decrease

in Try-nitration was observed on AOX OE. Taken together, these results were consistent with AOX modulating NO, O_2^- , $ONOO^-$ and Try-nitration.

AOX is producer of nitric oxide under hypoxic condition

Under hypoxic condition plant generate significant amount of NO [15], hence we tested how AOX could impact on NO generated during hypoxia. Seedlings were exposed to 0.4% oxygen for 6 hours immediately afterwards were incubated with DAF-FMDA and the relative fluorescence was assessed (Fig. 4)

Under normoxic conditions roots produced very little NO and this background NO production appeared to be further reduced in AOX OE roots. With AOX AS there was clear increase in NO levels compared to WT roots (Fig. 4A).

With hypoxia treatment, NO production in roots was elevated beyond anything detected under normoxic conditions (Fig 4A). In AOX OE, NO generation appeared to be more than that seen in WT roots but with AOX AS this was clearly reduced.. In order to confirm the possibility of AOX in generation of NO, seedlings were pre incubated with AOX inhibitor salicylhydroxamic acid (SHAM) for an hour and then these seedlings were exposed to hypoxia for 6 h and the NO production was measured using DAF-FM-DA. SHAM clearly inhibited hypoxia induced NO. Taken together our transgenic and pharmacological approaches clearly indicate that AOX is a generator of NO under hypoxia.

Higher AOX mediated NO production under hypoxia does not contribute to peroxynitrite formation

Given that AOX mediates increased NO production in response to hypoxia, we tested if O_2^- production and therefore $ONOO^-$ could be similarly influenced (Fig 5A).

In WT roots, the imposition of hypoxia did not appear to greatly increase O_2^- in compared to normoxic conditions. However, with the AOX OE line, there appear to be slight increase in O_2^- generation with hypoxia. The generation of O_2^- was much increased in the AOX AS in hypoxic over normoxic conditions. It should be noted that this is the inverse of pattern NO generation seen in Fig 4.

Analysis of $ONOO^-$ levels by APF fluorescence revealed that hypoxia leads to increased production of $ONOO^-$. Increased $ONOO^-$ was prominent in AOX AS and WT lines but AOX OE produced reduced levels of $ONOO^-$ (Fig 5B) despite of very high level of NO production (Fig 4A&B). Analysis of tyrosine nitration also revealed that AOX OE has reduced tyrosine nitration (Fig 5C). Taken together these results suggesting that increased levels of NO under hypoxic condition does not contribute significant towards $ONOO^-$ production. This could arise from increased NO generation not being matched with elevated O_2^- in hypoxia.

NO generation via AOX leads to increased energy efficiency under hypoxia

The AOX dependent increase in NO under hypoxia could feed into the non-symbiotic haemoglobin-NO cycle to contribute to the ATP generation. To test this possibility, we measured the expression of *non-symbiotic haemoglobin 1* (*nHb1*) transcript levels in response to hypoxia. Under hypoxic conditions *nHb1* expression has increased 180 fold in WT, 106 fold in AOX AS line where as strikingly 634 fold in the AOX OE line (Fig 6A). This indicated a correlation between NO and *nHb1* expression under hypoxia (compare Fig 4A and Fig 6A).

Other components of the haemoglobin-NO cycle, *NIA1, NIA2* expression, NR activity and ATP production were also tested. Assessments of *NIA1* expression indicated elevated expression in response to hypoxia but this did not significantly change

between WT, AOX OE and AOX AS genotypes (Fig 6B). In contrast to *NIA1*, the *NIA2* expression was supressed under hypoxia but again with no significant difference between the WT and AOX genotypes was observed (Fig 6C).

Since there is no change in expression of *NIA1* and *NIA2* between genotypes, we checked NR enzymatic activity. Under hypoxia a slight increase in NR activity was observed in WT and AS line but the OE line showed a 10-12 fold increase in NR activity. This suggested the active operation of Hb-NO cycle in AOX OE line and by implication with the increased NO seen in hypoxic WT lines.

The active operation of an AOX augmented Hb-NO cycle needed to be confirmed through increased ATP production. Measurements indicated significantly ($P < 0.05$) greater hypoxic ATP production in AOX OE over both WT and AOS-AS lines which did not significantly different from each other (Fig. 6E). To further confirm whether AOX induced NO feeds into Hb-NO cycle we checked ATP production in *nia1,2* mutant and found that this mutant has reduced ATP levels (Fig. 6E). To further confirm Hb-NO cycle involvement we measured ATP from non-symbiotic haemoglobin antisense line seedlings and found that this line has reduced ATP and the production of ATP was further diminished in the presence of SHAM suggesting that AOX mediated NO feeds into Hb-NO cycle.

Another prediction of an AOX augmented Hb-NO cycle is that it could confer increased plant viability under prolonged hypoxic stress. Thus, nine-day seedlings were maintained under hypoxic conditions (0.4% O_2) for 24 h and after a week they were assessed for relative viability. In the single example shown in Fig. 6G, the AOX OE seedlings appeared to be notably greener and therefore had a higher viability. This was quantified through measurements of chlorophyll content in the seedlings

(Fig. 6F). AOX OE seedlings had significantly ($P < 0.05$) higher chlorophyll content and additionally, this was significantly reduced in AOX AS. This latter result suggested reduced plant viability if the AOX augmented Hb-NO cycle is compromised under hypoxic conditions.

4. Discussion

NO has many roles in plant growth development and stress responses. Within a plant response to stress, it is an important signalling molecule that can confer tolerance [16-18] but equally can elicit programme cell death in plants [19]. One important mechanism through which NO can confer tolerance is via induction of AOX as a component of the mitochondrial ETC.

Several lines of evidence have unambiguously shown this to be the case and linked these events to stress tolerance. For instance, Huang et al. [12] demonstrated that NO treatment of Arabidopsis suspension cells increased the capacity of alternative respiratory pathway. Further, addition of an AOX inhibitor leads to increase NO sensitivity and cell death. Thus, NO induces the *AOX1a* gene to counteract the toxicity of NO. Another study demonstrated that pre-treatment of barley seedlings with NO increased both AOX1 expression and antioxidant enzyme activities thereby conferring better growth and adaptability during arsenic stress [20]. In a biotic stress, inoculation of lower leaves of tomato with tobacco mosaic virus (TMV) elicited a rapid increase in both NO and AOX in upper uninoculated leaves. This effect was directly associated with conferring resistance to disease as application of KCN (an inhibitor of cytochrome pathway) to upper uninoculated leaves, induced AOX transcripts and quantum yield of photosystem II and reduced the TMV viral accumulation [13].

These studies notwithstanding, most of these studies have not examined the roles of NO and AOX at sites of stress responses where the rates of NO and ROS production are considerable. One example of this would be the hypersensitive response (HR); a form of PCD associated with plant resistance to infection [19]. This situation has been previously explored by [21] who observed two different forms of HR one elicited by *Pseudomonas syringae* pv. (*P.s.*pv.) *phaseolicola* and another by *P. s.* pv.*maculicola. P. s.* pv. *Maculicola* elicited a substantial burst of mitochondrial O_2^- generation with no change in AOX, whilst *P.s.*pv. *phaseolicola* induced in AOX and no observable O_2^- generation in the mitochondria. The clear implication is that AOX regulates O_2^- generation during some forms of HR which agreed with the observed role of AOX in non-stressed plants previously studied by these same authors [7]. However, other than influencing the relative rate of cell death, AOX did not affect the ultimate outcome of the interaction; the formation of a HR [21].

In our case, we sought to focus on a component of the defence response which is also linked to NO and ROS, but not linked to the elicitation of cell death; so- called PAMP-triggered immunity (PTI) [22]. Bacterial flagellin and its conserved peptide flg22 is a well-characterised PAMP [23] and is known to elicit both ROS and NO [24,25,]. These responses to flg22 were confirmed in Arabidopsis seedlings and roots (Fig. 2, Fig.3).

To examine the roles of AOX in our studies we employed the well-characterised Arabidopsis lines where AOX expression is suppressed by and anti-sense approach (AOX AS) and also where AOX was over-expressed (AOX OE) [26]. Our characterisation of the responses of WT, AOX AS and AOX OE to flg22 demonstrated an inverse correlation between the rates of NO and O_2^- generation

and AOX expression. This accorded with well-established role of AOX in suppressing NO and ROS under normoxia in unstressed plants [21].

Beyond this we examined the potential role of AOX influenced events following flg22 elicitation. Flg22 is an effective inducer of AOX expression (Fig. 1) and it is likely that one of its role is to dampen ROS, NO accumulation (Fig. 2 and Fig 3) and thereby reduce $ONOO^-$ generation (Fig. 3B) and tyrosine nitration (Fig. 3C). This could be important in maintaining plant cell viability during PTI. In this context, it is relevant that increasing tyrosine nitration has been observed with the senescence form of cell death [27]. Many nitrated proteins were detected in this study analysis of these proteins would give new insights for the role of these nitrated proteins under hypoxia and reoxygenation. We also investigated previously unexplored links between hypoxia, NO and AOX. Previously it was demonstrated that Complex III and cytochrome c oxidase are the sites for NO production [4,15] but the contribution of AOX in hypoxic/anoxic NO was largely ignored. Most plants need to respond to hypoxic stress at various points in their lifecycles. Mostly obviously, heavy rain or flooding will result in soil water logging will limit the ability of oxygen to diffuse to the roots [28]. Plants may employ one of many physiological responses to submergence including hyponasty, elongation of floral stems, petioles or internodes or even the formation of aerial roots [29]. Many of the mechanisms underlying these responses to low oxygen have been extensively characterised [30] and some have been shown to be influenced by NO [31]. Given this background, it was entirely appropriate that we explored hypoxic NO, AOX effects in roots. This stated, hypoxic responses are also features of normal seed development [32].

Firstly, we demonstrated that AOX transcript and protein are clearly upregulated under hypoxia in seedlings (Fig 1) and roots (Supplementary Figure 1). Our study

explored several roles for this increased AOX using the transgenic AOX lines. As we focused on roots, we established that the patterns of AOX expression in the lines were similar to those reported in leaves (Supplementary Figure 1). We initially explored that AOX could modulate hypoxic ROS generation and therefore the plant post-stress survival [33]. Our analyses demonstrated increased O_2^- (Fig 5) in response to hypoxic treatment and these are suppressed by AOX, as noted in normoxic situations. However, our important observation as that under hypoxia results providing AOX itself can produce NO, whereas same protein scavenges NO under normoxia. These data clearly indicated a discrete role for AOX under hypoxia. Interestingly, hypoxic roles for AOX did not extent to H_2O_2 which was also elevated (Supplementary Fig. 2). This indicated non-AOX mediated forms of ROS generation during limited O_2 supply.

The role of the NO could be to inhibit aconitase increased citrate which can provide carbon skeletons for amino acid synthesis which is important for hypoxic survival [11]. Equally, the role of AOX increasing NO (O_2^- + NO ➔ $ONOO^-$) could act to reduce the formation of $ONOO^-$. Our results show that AOX OE line has increased levels of NO (Fig 4) yet reduced levels of $ONOO^-$ and tyrosine nitration (Fig 5) suggesting that in the presence of AOX toxic $ONOO^-$ formation can be reduced either due to scavenging of excess levels of O_2^- by AOX. A complementary hypothesis could be that there could be competition for NO between nHb1 and O_2^- for the formation of $ONOO^-$. This would immediately imply that and AOX augmented Hb-NO cycle is important under hypoxia, possibly preventing the formation of $ONOO^-$ but also contributing to ATP generation. Further, NO can inhibit COX but AOX is not sensitive to NO. Thus, if excess of NO produced under hypoxic conditions it can

prevent COX to function. Under such conditions AOX induction can drive anaerobic ATP synthesis [34].

Our subsequent analyses explored the possibility of an AOX augmented Hb-NO cycle in hypoxia (Fig. 7). We found increases in each of the key players in this cycle under hypoxia. Thus, AOX dependent generation of NO was demonstrated using transgenic lines and also via inhibition using SHAM (Fig. 4). This should feed into a NO oxidation step mediated by nHb.

Other authors have previously demonstrated that NO and hypoxia induces *nHb1* [9]. In this study, the increased expression of *nHb1* in the AOX OE line (Fig. 6A) demonstrated how AOX generated NO fed into nHb1. The NO_3^- product should then be reduced back to NO through the combined action nitrate NR and nitrite: NO reductase (Ni:NOR) reactions. In our study we found increased NR enzymatic activity in response to hypoxia (Fig 6D) although but we found no significant change in *NIA1* expression in WT, AOX AS and AOXOE lines. This suggests that hypoxic NR activity was being modified through established post-translation modifications [35] such as phosphorylation or interactions with 14-3-3 proteins [36]. It could be a direct effect of NO itself as this can stimulate the activity of NR in plant roots [37]. In contrast to *NIA1*, the *NIA2* transcript levels were supressed under hypoxia via AOX and therefore mostly likely NO independent mechanisms (Fig, 6C). Therefore, our observed increase in NR activity (Fig. 6D) could be a mediated by the *NIA1* a NR product. *NIA2* encodes the NR form which contributes around 90 % of a plants NR activity [38], so its reduced expression could imply that the plants nitrate assimilatory capacity would affected. This is currently being assessed in our group.

The main predication of our model is that AOX would contribute to increased ATP generation during hypoxia. This was suggested from our direct ATP measurements in WT and the AOX transgenic lines, *nia 1,2* mutant and nHb1 silencing line (Fig. 6E). Further, AOX and presumably, at least in part, in the augmented Hb/NO cycle clearly contributes to increased tolerance to hypoxia (Fig. 6F&G).

Taken together our results demonstrate a novel role of AOX in NO metabolism in plants under hypoxia. Previously, it was thought AOX contributed to energetically wasteful processes but in hypoxia AOX itself can contribute indirectly for increasing energy efficiency by reduction of nitrite to NO and pushing Hb-NO cycle. Much research effort is focused on the derivation of flood tolerant varieties of; for example, rice. Thus, genes such as *Submergence 1A* (*SUB1A*) and stem elongating *SNORKEL1* and *SNORKEL2* have been introgressed to high-yielding rice varieties [39]. Our data shows that AOX OE line survived much better in response to hypoxic stress (Fig 6) suggesting that AOX over expressing in crop plants could be another target in breeding programmes targeting flooding/water logging tolerance.

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Conflict of interest

The authors have no conflicts of interest with the contents of this article.

Author contributions

KJG conceived original research plans. AV performed the experiments. LM provided AOX transgenics, AOX antibodies. KJG and LM prepared the manuscript.

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Supplementary Figure 1. Expression of AOX in the root of WT Arabidopsis roots and of alternative oxidase over-expressing (AOX OE) and anti-sense (AOX AS) transgenic lines.

For immunodetection ofAOX1a protein, total protein (50 µg) from each sample was separated on 10% SDS-PAGE and followed by detected using anti-AOX1a antibody as described in methodology.

Supplementary Figure 2. Changes in H_2O_2 in WT, AOX1a antisense (AOX AS) and AOX1a over-expression (AOX OE) lines in response to 6 h hypoxia treatment.

Microscopic detection of H_2O_2in Arabidopsis roots following staining with 3, 3-diaminobenzidine (DAB). Under normoxia and after hypoxia treatment, roots were

incubated with 0.1% DAB solution for 2 h at room temperature under dark conditions. Images were taken with the light microscope at 20X zoom.

Supplementary Table 1. The list of primers used in the present study for qRT-PCR analysis.

References:

[1] G.C.Vanlerberghe,Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int J Mol Sci 14(2013) 6805–6847.

[2] K.J.Gupta, A.R.Fernie, W.M.Kaiser, J.T.van Dongen, On the origins of nitric oxide. Trends Plant Sci 16(2011) 160–168.

[3] M.Moreau, C.Lindermayr, J.Durner, D.F.Klessig,NO synthesis and signaling in plants--where do we stand? Physiol Plant 138(2010)372–383.

[4] K.J.Gupta, M.Stoimenova, W.M.Kaiser,In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. J Exp Bot 56(2005) 2601–2609.

[5] N.A.Alber, H.Sivanesan, G.C.Vanlerberghe,The occurrence and control of nitric oxide generation by the plant mitochondrial electron transport chain. Plant Cell Environ 40(2017) 1074–1085.

[6] K.J.Gupta, W.M.Kaiser,Production and scavenging of nitric oxide by barley root mitochondria. Plant Cell Physiol 51(2010) 576–584.

[7] M.Cvetkovska, G.C.Vanlerberghe,Alternative oxidase modulates leaf

mitochondrial concentrations of superoxide and nitric oxide. New Phytol 195(2012) 32–39.

[8] S.Amirsadeghi, C.A.Robson, A.E.McDonald, G.C.Vanlerberghe,Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signalling molecules. Plant Cell Physiol. 47(2006)1509-1919.

[9] A.U.Igamberdiev, N.V.Bykova, R.D.Hill,Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin. Planta 223(2006) 1033–1040.

[10] M.Stoimenova, A.U.Igamberdiev, K.J.Gupta, R.D.Hill,Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. Planta 226(2007) 465–474.

[11] B.Royo, J.F. Moran, R.G. Ratcliffe,K.J. Gupta, Nitric oxide induces the alternative oxidase pathway in Arabidopsis seedlings deprived of inorganic phosphate. J Exp Bot 66(2015) 6273–80.

[12] X.Huang, U.von Rad, J.Durner,Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in Arabidopsis suspension cells. Planta 215(2002) 914–923.

[13] L-J.Fu, K. Shi, M.Gu, Y-H.Zhou, D-K.Dong, W-S.Liang, F-M.Song, J-Q.Yu,Systemic induction and role of mitochondrial alternative oxidase and nitric oxide in a compatible tomato-Tobacco mosaic virus interaction. Mol Plant Microbe Interact 23(2010) 39–48.

[14] M.C.Palmieri, C.Lindermayr, H.Bauwe, C.Steinhauser, J.Durner,Regulation of plant glycine decarboxylase by s-nitrosylation and glutathionylation. Plant

Physiol 152(2010) 1514–1528.

[15] E.Planchet, K.J.Gupta, M.Sonoda,W.M.Kaiser, Nitric oxide emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J 41(2005) 732–743.

[16] M.H.Siddiqui, M.H.Al-Whaibi, M.O.Basalah,Role of nitric oxide in tolerance of plants to abiotic stress. Protoplasma 248(2011) 447–455.

[17] S.Sidana, J.Bose, L.Shabala, S.Shabala,Nitric Oxide in Drought Stress Signalling and Tolerance in Plants. *In* MN Khan, M Mobin, F Mohammad, FJ Corpas, eds, Nitric Oxide Action Abiotic Stress Responses Plants. Springer International Publishing, Cham, (2015) pp 95–114.

[18] D.Arora, P.Jain, N.Singh, H.Kaur, S.C.Bhatla,Mechanisms of nitric oxide crosstalk with reactive oxygen species scavenging enzymes during abiotic stress tolerance in plants. Free Radic Res 50(2016) 291–303.

[19] H-Y.He, M-H.Gu, L-F.He,The Role of Nitric Oxide in Programmed Cell Death in Higher Plants. *In* MN Khan, M Mobin, F Mohammad, FJ Corpas, eds, Nitric Oxide Plants Metab. Role Stress Physiol. Springer International Publishing, Cham, (2014) pp 281–296.

[20] P.Shukla, S.Singh, P.Dubey, A.Singh, A.K.Singh,Nitric oxide mediated amelioration of arsenic toxicity which alters the alternative oxidase (Aox1) gene expression in Hordeum vulgare L. Ecotoxicol Environ Saf 120(2015) 59–65.

[21] M.Cvetkovska, G.C.Vanlerberghe,Alternative oxidase impacts the plant response to biotic stress by influencing the mitochondrial generation of reactive oxygen species. Plant Cell Environ 36(2013) 721–732.

[22] J.Monaghan, C.Zipfel,Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol 15(2012) 349–357.

[23] Y.Rossez, E.B.Wolfson, A.Holmes, D.L.Gally, N.J.Holden,Bacterial flagella: twist and stick, or dodge across the kingdoms. PLoS Pathog 11(2015)e1004483.

[24] D.Zeidler, U.Zahringer, I.Gerber, I.Dubery, T.Hartung, W.Bors, P.Hutzler, J.Durner,Innate immunity in Arabidopsis thaliana: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc Natl Acad Sci U S A 101(2004) 15811–15816.

[25] Y.Sang, A.P.Macho,Analysis of PAMP-Triggered ROS Burst in Plant Immunity. Methods Mol Biol 1578(2017) 143–153.

[26] F.Fiorani, A.L.Umbach, J.N.Siedow,The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139(2005) 1795–1805.

[27] J.C.Begara-Morales, M.Chaki, B.Sanchez-Calvo, C.Mata-Perez, M.Leterrier, J.M.Palma, J.B.Barroso, F.J.Corpas,Protein tyrosine nitration in pea roots during development and senescence. J Exp Bot 64(2013) 1121–1134.

[28] S.Lekshmy, S.K.Jha, R.K.Sairam,Physiological and Molecular Mechanisms of Flooding Tolerance in Plants. *In* GK Pandey, ed, Elucidation Abiotic Stress Signal. Plants Funct. Genomics Perspect. Vol. 2. Springer New York, New York, NY, (2015) pp 227–242.

[29] L.A.C.J.Voesenek, J.Bailey-Serres,Flood adaptive traits and processes: an overview. New Phytol 206(2015) 57–73.

[30] J.Bailey-Serres, L.A.C.J.Voesenek,Life in the balance: a signaling network controlling survival of flooding. Curr Opin Plant Biol 13(2010) 489–494.

[31] K.H.Hebelstrup, M.Van Zanten, J.Mandon, L.A.C.J.Voesenek, F.J.M.Harren, S.M.Cristescu, I.M.Møller, L.A.J.Mur,Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in Arabidopsis thaliana. J Exp Bot 63(2012) 5581–5591.

[32] M.Gayral, K.Elmorjani, M.Dalgalarrondo, S.M.Balzergue, S.Pateyron, M-H.Morel, S.Brunet, L.Linossier, C.Delluc, B.Bakan, et al, Responses to Hypoxia and Endoplasmic Reticulum Stress Discriminate the Development of Vitreous and Floury Endosperms of Conventional Maize (Zea mays) Inbred Lines. Front Plant Sci 8(2017) 557.

[33] B.Steffens, A.Steffen-Heins, M.Sauter,Reactive oxygen species mediate growth and death in submerged plants. Front Plant Sci.(2013) doi: 10.3389/fpls.2013.00179.

[34] A.H.Millar, D.A.Day,Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Lett 398(1996) 155–158.

[35] P.Rockel, F.Strube, A.Rockel, J.Wildt, W.M.Kaiser,Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J Exp Bot 53(2002) 103–110.

[36] W.H.Campbell,Structure and function of eukaryotic NAD(P)H:nitrate reductase. Cell Mol Life Sci 58(2001) 194–204.

[37] C.W.Jin, S.T.Du, Y.S.Zhang, X.Y.Lin, C.X.Tang,Differential regulatory role of nitric oxide in mediating nitrate reductase activity in roots of tomato (Solanum

lycocarpum). Ann Bot 104(2009) 9–17.

[38] J.Q.Wilkinson, N.M.Crawford,Identification and characterization of a chlorate-resistant mutant of Arabidopsis thaliana with mutations in both nitrate reductase structural genes NIA1 and NIA2. Mol Gen Genet 239(1993) 289–297.

[39] S.K.Singh, C-T.Chien, I-F.Chang,The Arabidopsis glutamate receptor-like gene GLR3.6 controls root development by repressing the Kip-related protein gene KRP4. J Exp Bot 67(2016) 1853–1869.

[40] N.Jambunathan,Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. Methods Mol Biol 639(2010) 292–298.

[41] K.J.Livak, T.D.Schmittgen,Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(2001) 402–408.

[42] U.K.Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (1970) 680-685.

Figure 1. AOX1a transcript and protein analysis in WT seedlings of *Arabidopsis thaliana* in response to normoxia, normoxia + 2.5 µM flg22 and 6h hypoxia. (A) Transcript levels were calculated using ΔΔCt method.18S rRNA was used as housekeeping gene. (B) For immunodetection of AOX1a protein, total protein (50 µg) from each sample was separated on 10% SDS-PAGE and followed by detected using anti-AOX1a antibody as described in methodology. Band intensity was calculated using Image J software.

Figure 2. Microscopic NO detection in the roots of WT, AOX1a antisense lines (AOX AS) and AOX1a overexpression lines (AOX OE) in response to normoxia and 2.5 µM flg22. (A) Two weeks old seedlings were incubated with 10 µM DAF for 20 min at room temperature and observed in a fluorescence microscope with excitation/emission maxima of 495/515 nm. (B) DAF fluorescence was quantified in 20 seedling per treatment using Image J software. Asterisks indicate significant difference compared to WT under normoxic or hypoxic conditions ($P<0.05$). Bar = 50 µm

Figure 3. Changes in O_2^-, $ONOO^-$ and tyrosine nitrated proteins in WT, AOX1a antisense (AOX AS) and AOX1a over-expression (AOX OE) lines in response to flg22 under normoxic conditions. (A) Microscopic O_2^- detection in Arabidopsis roots. After flg22 treatment, roots were incubated with 0.1% NBT for 24 h and images were taken in light microscope at 20X zoom. The extent of straining was quantified in 20 seedling per treatment using Image J software (Right panel). The letters indicate significant (P <0.05) groupings. (B) $ONOO^-$ (APF fluorescence) detection in Arabidopsis roots. After flg22 treatment, roots were incubated with 10 µM APF in 10

mM HEPES pH 7.2 for 20 min at room temperature and observed in fluorescence microscope using λ_{488} excitation and $\lambda_{500\text{-}530}$ emission wavelength. Fluorescence was quantified by fluorimeter (Right panel). The letters indicate significant (P <0.05) groupings. (C) Immunodetection of tyr-nitrated proteins. Total protein was extracted from seedlings treated with 2.5 µM flg22. Total protein equivalent to 25 µg was separated on 10% SDS-PAGE under non-reducing condition (Ponceau stain was displayed as loading control). Membrane was treated with Anti-tyr-nitrosine antibody (1:2000) and secondary antibody IgG goat anti-rabbit HRP conjugate (1:5000). The blot was developed using chemiluminescent substrate. The band intensity was quantified using Image J software (Right panel). The blot is a representative image of 3 independent replicates.

Figure 4. Microscopic NO detection in the roots of WT, AOX1a antisense lines (AOX AS) and AOX1a overexpression lines (AOX OE) under normoxic and hypoxic conditions. (A) Two week old seedlings under normoxic or hypoxic conditions or hypoxic conditions with 2.5 mM SHAM were incubated with 10 µM DAF-FM-DA for 20 min at room temperature and observed in a fluorescence microscope with excitation/emission maxima of 495/515 nm. (B) DAF fluorescence was quantified in 20 seedling per treatment using Image J software. The letters indicate significant (P <0.05) groupings.

Figure 5. Changes in O_2^-, $ONOO^-$ and tyrosine nitrated proteins in WT, AOX1a antisense (AOX AS) and AOX1a over-expression (AOX OE) lines in response to 6 h hypoxia treatment. (A) Microscopic O_2^- detection in Arabidopsis roots. After hypoxia treatment, roots were incubated with 0.1% NBT for 24 h and images were taken with the light microscope at 20X zoom. The extent of straining was quantified in 20 seedling per treatment using Image J software (Right panel). The letters

indicate significant (P <0.05) groupings. (B) Measurement of $ONOO^-$ (APF fluorescence) levels in Arabidopsis roots. After hypoxia treatment, roots were incubated with 10 µM APF in 10 mM HEPES pH 7.2 for 20 min at room temperature and observed under a fluorescence microscope using λ_{488} excitation and $\lambda_{500-530}$ emission wavelength. Fluorescence was quantified by fluorimeter (Right panel). . The letters indicate significant (P <0.05) groupings. (C) Immunodetection of tyr-nitrated proteins. Total protein was extracted from seedlings treated with normoxia and hypoxia. Total protein equivalent to 25 µg was separated on 10% SDS-PAGE under non-reducing condition (Ponceau stain was displayed as loading control). The membrane was treated with Anti-tyr-nitrosine antibody (1:2000) and secondary antibody IgG goat anti-rabbit HRP conjugate (1:5000). The blot was developed using chemiluminescent substrate. The band intensity was quantified using Image J software. The blot is a representative image of 3 independent replicates. WT band intensity was normalised to 100% and compared with the intensity of AS and OE for each treatment.

Figure 6. Effect of hypoxia treatment on Hb/NO cycle, energy efficiency and survival in the seedlings of WT, AOX1a antisense (AS) and AOX1a overexpression (OE) lines. (A-C) Changes in *HB1*, *NIA1* and *NIA2* transcript levels in response to normoxia and 6 h hypoxia. Transcript levels were calculated using ΔΔCt method and *18S rRNA* was used as housekeeping gene; The letters indicate significant (P <0.05) groupings.(D) Measurement of nitrate reductase (NR) activity in response to normoxia and 6 h hypoxia; The letters indicate significant (P <0.05) groupings. (E) Measurement of ATP levels after 6 h hypoxia treatment employed a luciferase based assay kit; The letters indicate significant (P <0.05) groupings (F) Total chlorophyll content from reoxygenated seedlings following hypoxia as measured by a

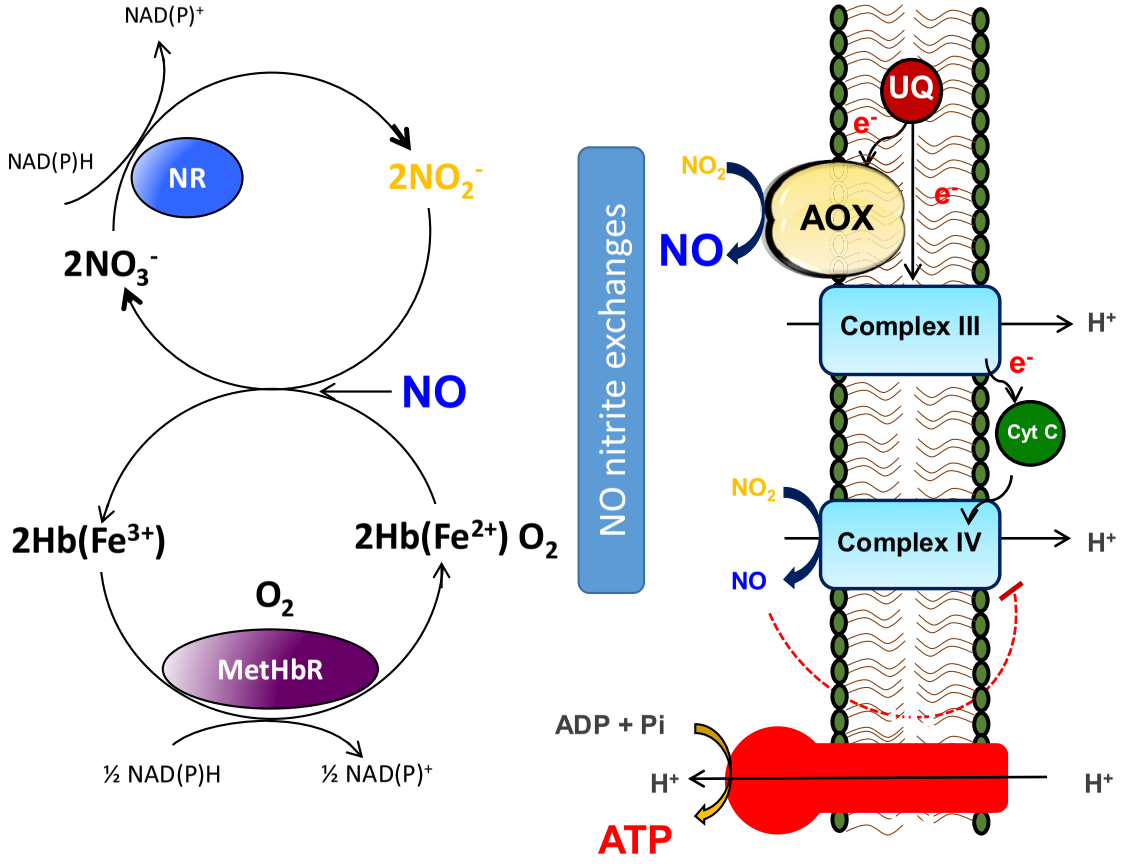
colorimetric assay. Values presented here are the mean (n = 3±SD). (G) Phenotypes of seedlings before, and after exposure to 24 h hypoxia treatment and seven days after reoxygenation.

Figure 7. Schematic model showing alternative oxidase generated NO feeding into the haemoglobin-NO cycle during hypoxia. Under oxygen limiting conditions, cytosolic NR produces very high amounts of NO due to inhibition of NO_2^-reduction to NH_4^+ is inhibited [15]. Excess of NO will contribute to the inhibition of cytochrome c oxidase or Complex IV (dotted red line) which can have NO consuming activities. We suggest when COX is inhibited the increased NO_2^- is reduced to NO by alternative oxidase (AOX) under conditions of hypoxia. The elevated NO will feed into the haemoglobin-NO cycle. The haemoglobin–nitric oxide (Hb/NO) cycle has been described elsewhere [10] but briefly involved NO scavenging by oxyhemoglobin ($Hb(Fe^{2+})O_2$) which is reduced to $2Hb(Fe^{3+})$ by the methemoglobin reductase (MetHb-R) protein. This is coupled to NO oxidation to from NO_3^-. Nitrate reductase (NR) will produce NO_2^- to serve as an electron donor at AOX which are linked to the mitochondrial electron transport chain [4,15] and ATP synthesis. Note the reductive power is provided by NAD(P)H at several steps.

Highlights

- Under normoxic conditions AOX acts as scavenger of NO when treated with flg22
- AOX over expressing line (AOX OE) displayed less NO and opposing effect was observed in AOX antisense line (AOX-AS) under normoxia in response to flg22 treatment
- Scavenging of NO by AOX can prevent toxic peroxynitrite and tyrosine nitration formation under normoxia
- In contrast to normoxia, during hypoxia AOX is a generator of NO
- The enhanced levels of NO under hypoxia correlated with expression of non-symbiotic haemoglobin, increased NR activity and ATP production
- Hypoxic NO generation mediated by AOX has a discrete role by feeding into the haemoglobin-NO cycle to drive energy efficiency under conditions of low oxygen tension.

Figure 7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NAD(P)H
NR
2NO2-
2NO3-
NO
2Hb(Fe3+)
2Hb(Fe2+) O2
O2
MetHbR
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½ NAD(P)+
NO nitrite exchanges
NO2
NO
AOX
UQ
e-
e-
Complex III
H+
e-
Cyt C
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Complex IV
H+
NO
ADP + Pi
H+
H+
ATP

Figure 6

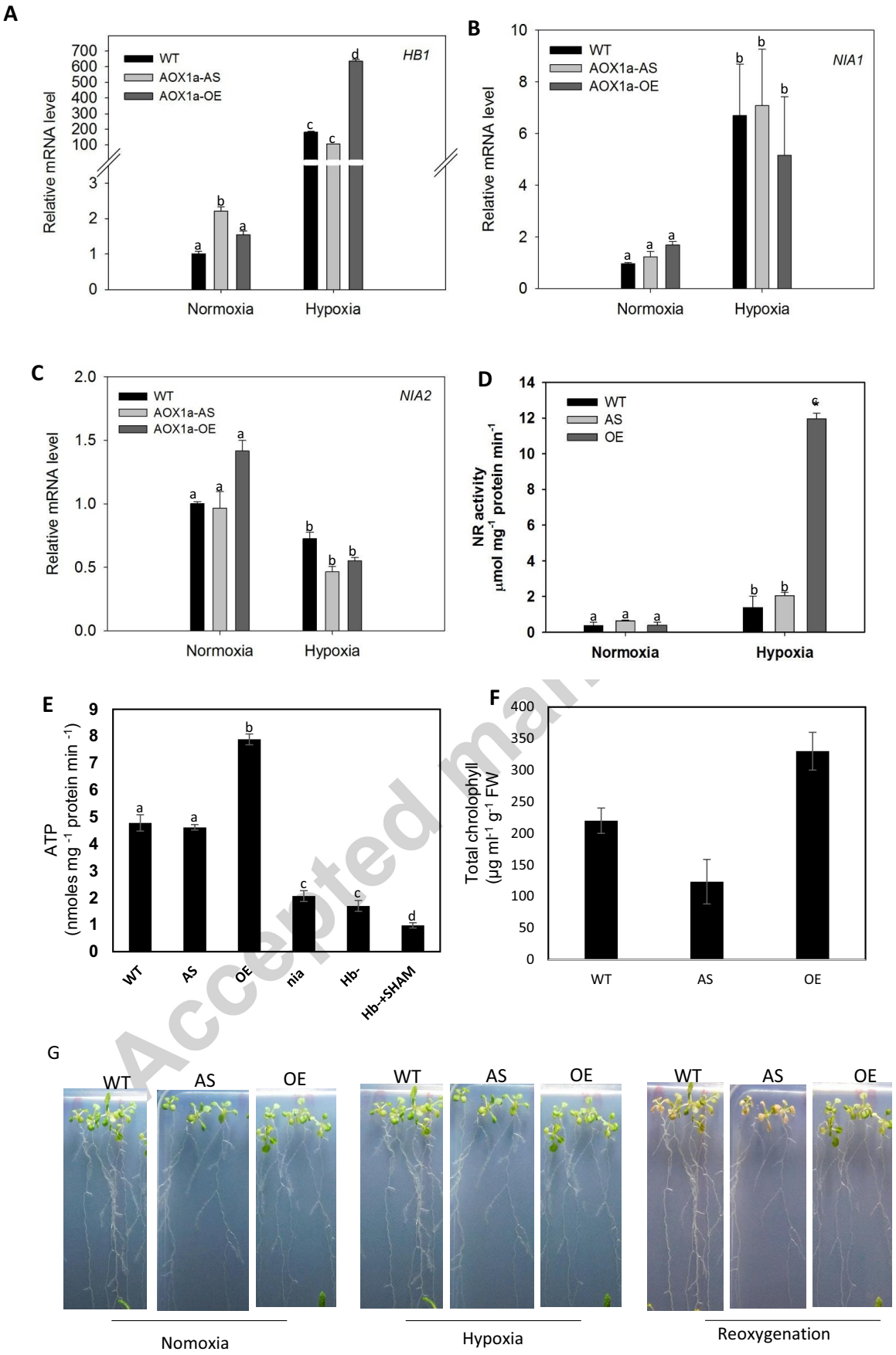


Figure 5

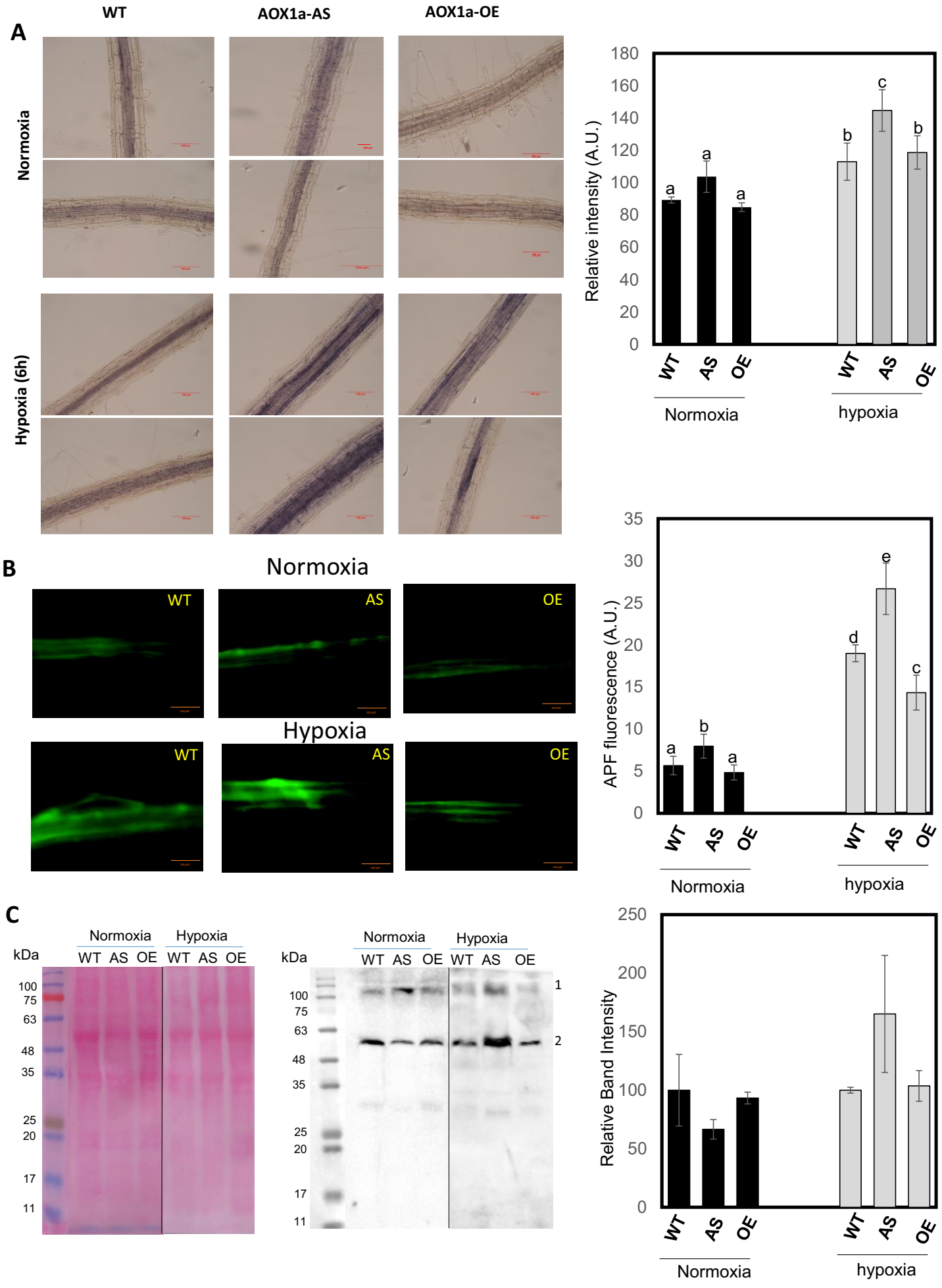

A
WT
AOX1a-AS
AOX1a-OE
Normoxia
Hypoxia (6h)
Relative intensity (A.U.)
Normoxia
hypoxia
B
Normoxia
WT
AS
OE
Hypoxia
APF fluorescence (A.U.)
C
kDa
Normoxia
Hypoxia
Relative Band Intensity

Figure 4

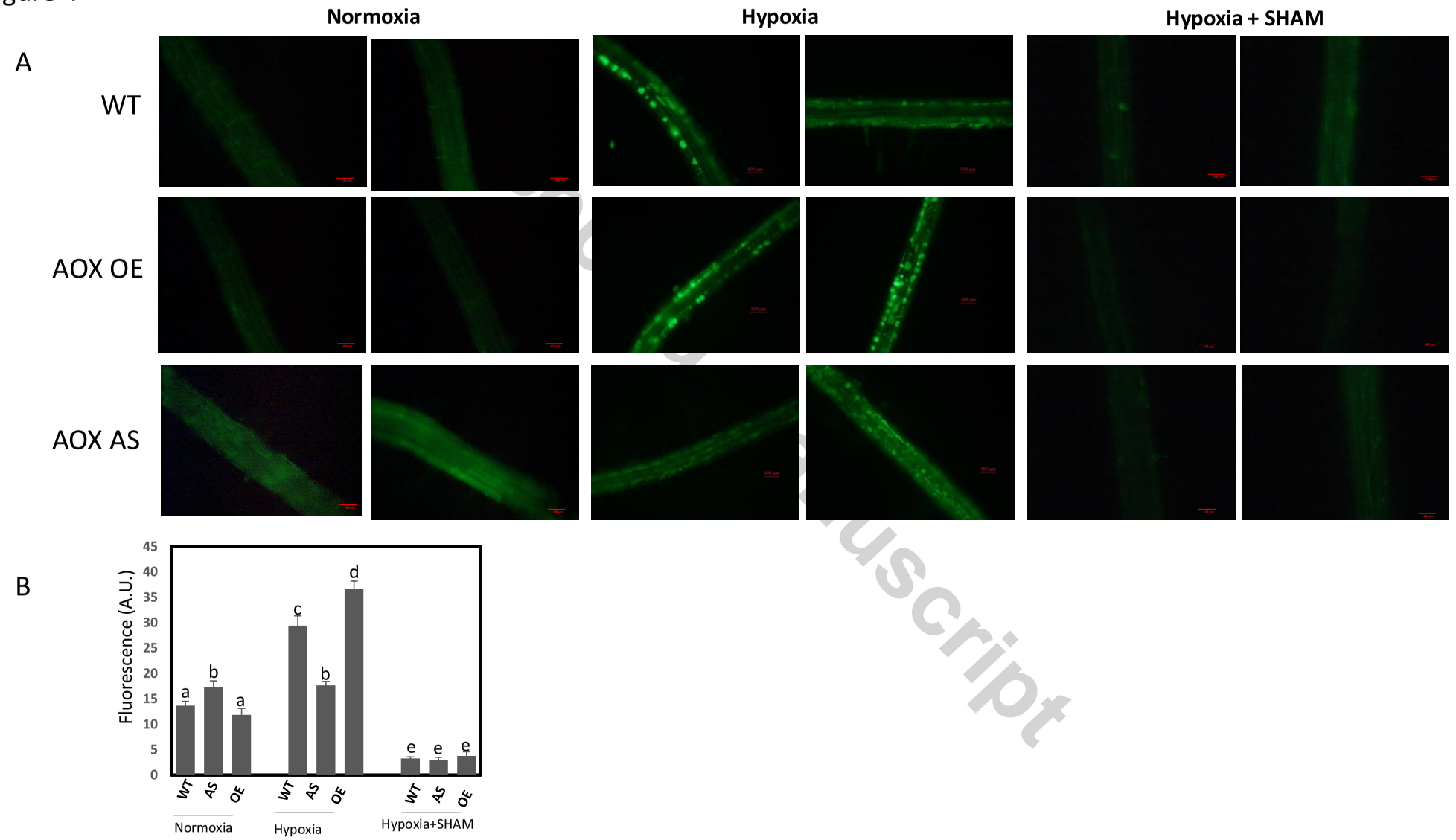
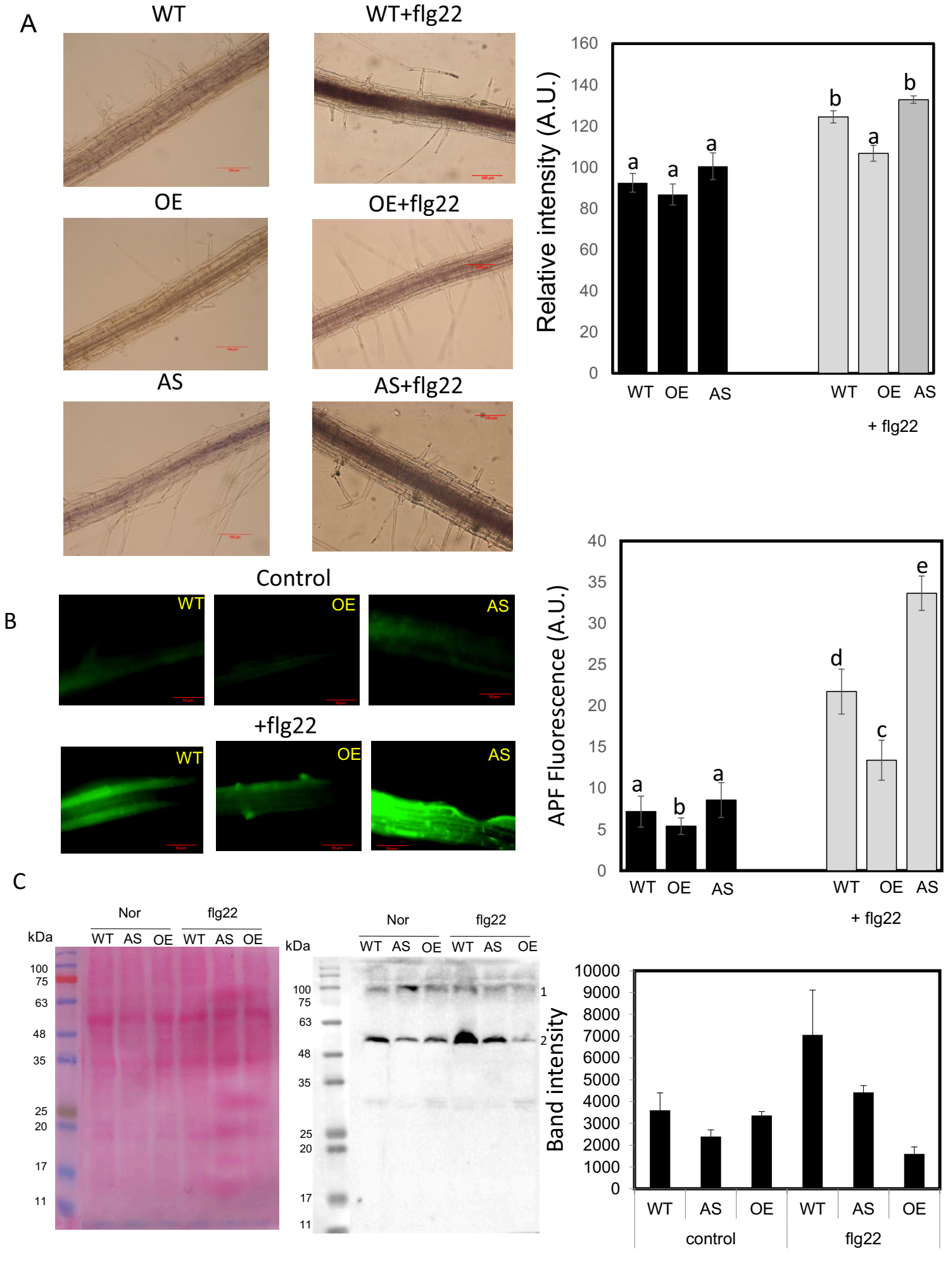


Figure 3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WT
WT+flg22
OE
OE+flg22
AS
AS+flg22
Relative intensity (A.U.)
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140
120
100
80
60
40
20
0
a
a
a
b
a
b
WT OE AS
WT OE AS
+ flg22
B
Control
WT
OE
AS
+flg22
WT
OE
AS
APF Fluorescence (A.U.)
40
35
30
25
20
15
10
5
0
a
b
a
d
c
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WT OE AS
WT OE AS
+ flg22
C
Nor
flg22
kDa
WT AS OE WT AS OE
100
75
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48
35
25
20
17
11
1
2
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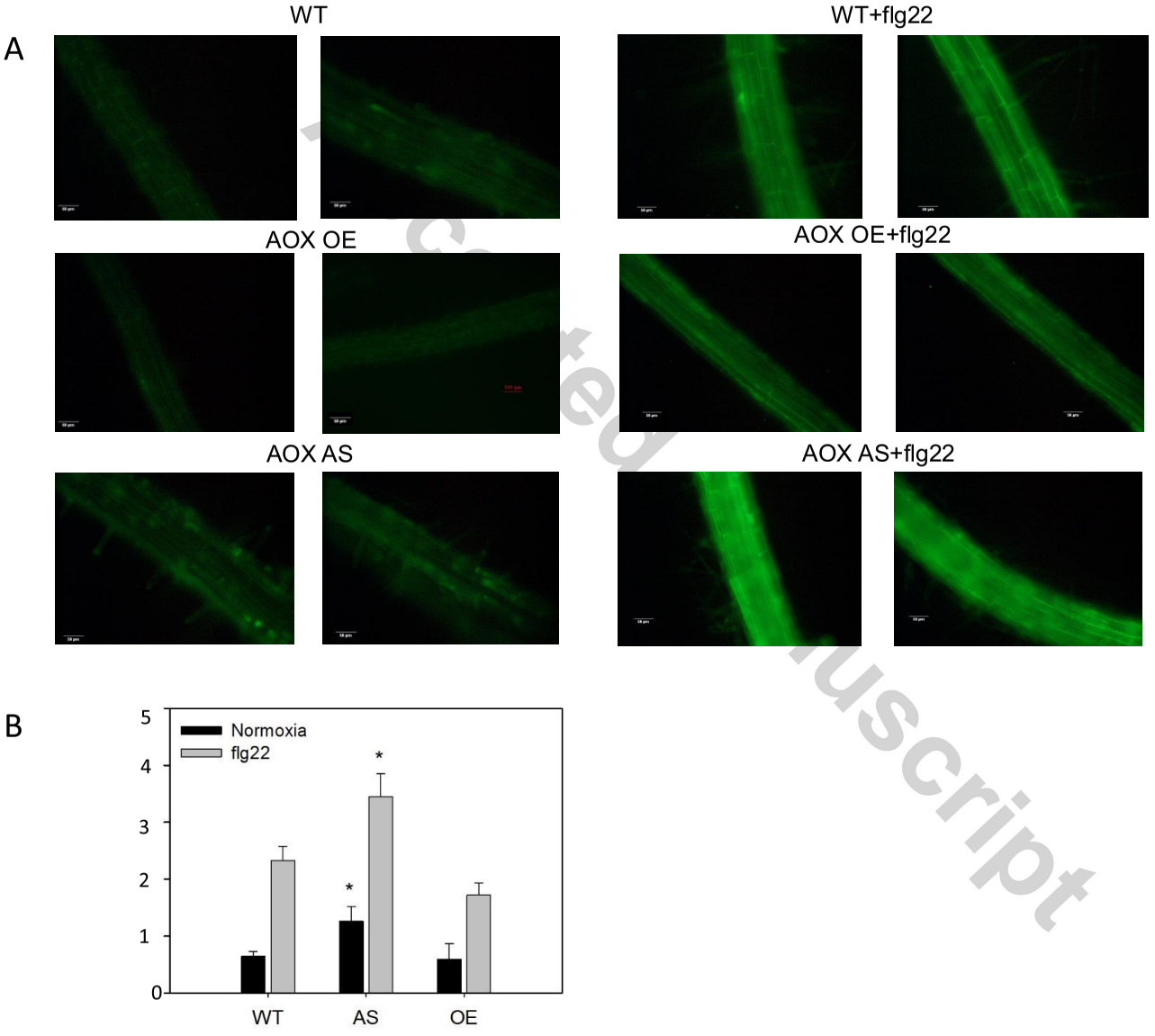
control
flg22

Figure 2



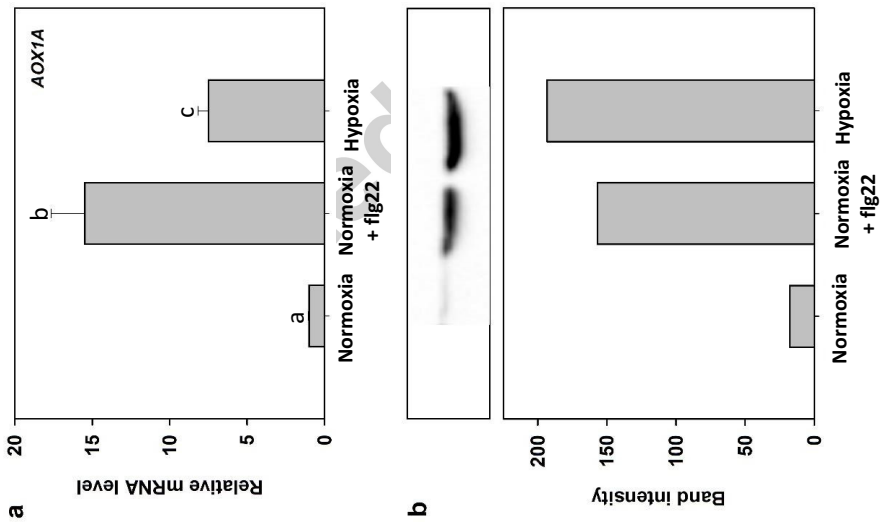


Figure 1